Visceral antinociception produced by bee venom stimulation of the Zhongwan acupuncture point in mice: role of $\alpha_2$ adrenoceptors

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Abstract
The goal of the present study was to determine whether bee venom (BV) injection into the Zhongwan acupoint (CV12), compared to injection into a non-acupoint, produced antinociception in an acetic acid-induced visceral pain model. This was accomplished by injecting BV subcutaneously into the Zhongwan acupoint or into a non-acupoint 30 min before intraperitoneal injection of acetic acid in ICR mice. BV injection into the acupoint produced a dose dependent suppression of acetic acid-induced abdominal stretches and of acetic acid-induced Fos expression in the spinal cord and the nucleus tractus solitarii. In contrast BV injection into the non-acupoint only produced antinociception at the highest dose of BV tested. Naloxone pretreatment did not alter the antinociceptive effect of BV acupoint injection on the abdominal stretch reflex. On the other hand, pretreatment with the $\alpha_2$-adrenoceptor antagonist, yohimbine completely blocked the antinociceptive effect of BV acupoint injection. These results imply that BV acupoint stimulation can produce visceral antinociception that is associated with activation of $\alpha_2$-adrenoceptors, but not with naloxone-sensitive opioid receptors.

Keywords: Bee venom; Antinociception; Acupuncture; Abdominal nociception; Fos expression; Adrenoceptor antagonist

Traditionally acupuncture has been the method of choice in oriental medicine for the production of analgesic effects in the treatment of a variety of pain producing disease processes. While the mechanisms that contribute to acupuncture analgesia are still under investigation, it is generally accepted that acupuncture point (acupoint) stimulation produces a more potent analgesic effect than stimulation of a non-acupoint and that the analgesia produced can be modified by the type of stimulation that is applied. Although acupoint activation has been traditionally induced by mechanical or electrical stimulation, we hypothesize that acupoint can also be effectively activated by chemical stimulation.

We have recently demonstrated that the administration of bee venom (BV) directly into an acupoint produces a significantly more potent antinociceptive effect than that observed following injection into a non-acupoint in a model of adjuvant-induced arthritis and in a model of tonic pain (the formalin test) [8,9]. Because subcutaneous injection of BV chemically activates the injected area and appears to initiate stimulation of nerve fibers, particularly nociceptors, in the region [1,10], we hypothesized that direct injection of BV into an acupoint would result in acupoint stimulation and that this stimulation underlies the BV-induced antinociception and anti-inflammation that we have observed previously in rodent models of arthritis and formalin test.

From a neurochemical standpoint, both clinical and experimental data suggest that the opioid system and the adrenergic system play important roles in acupuncture-induced analgesia in somatic pain states [12]. On the other hand, the potential involvement of these systems in the generation of acupuncture analgesia in the treatment of visceral pain has not been evaluated. While it is unclear if these systems are involved in acupuncture-induced visceral pain relief, it is well established that opioid agonists such as
morphine have a potent antinociceptive effect in animal models of visceral pain and that this effect is antagonized by naloxone [14]. Similarly, it has been reported that the \(\alpha\)-adrenoceptor agonist, clonidine also produces antinociception in a visceral pain model and this antinociceptive effect is completely antagonized by treatment with the \(\alpha_2\)-adrenoceptor antagonist, yohimbine, but not with the \(\alpha_1\)-adrenoceptor antagonist, prazosin [13]. Thus, activation of opioid and adrenergic components associated with central descending antinociceptive pathways have been postulated to be involved in the analgesic action of opioid and \(\alpha_2\)-adrenergic agonists in visceral pain models [3]. However, it is not known whether central opioid and \(\alpha_2\)-adrenergic components of the intrinsic descending analgesic system are activated during acupuncture treatment of visceral nociception. To examine this issue in the present study, we injected naloxone or yohimbine subcutaneously prior to BV acupuncture treatment in order to determine whether the antinociceptive effect of BV acupoint injection on visceral nociception involved central opioid and/or adrenergic components.

Experiments were performed on male ICR mice, weighing 32–36 g. All experimental animals were provided by the Laboratory Animal Center of Seoul National University (SNU) and the experimental protocols for animal usage were reviewed and approved by the SNU Animal Care and Use Committee.

The mice were placed individually in table-top plexiglass observation cylinders (60 cm high; 40 cm diameter) and were allowed to adapt to the environment for 30 min prior to the start of the experiment. Acetic acid (0.6 ml of a solution at a concentration of 0.9% v/v) was injected intraperitoneally to produce abdominal stretches. The number of abdominal stretches per animal was recorded over the next 30 min using a video camera. Following the videotaping, the number of abdominal stretches was counted by two experienced investigators blinded to the experimental conditions.

In the first experiment, we evaluated whether injection of BV into an acupoint produced a greater antinociceptive effect than injection of BV into a non-acupoint employing the abdominal stretch assay in mice. In order to test the antinociceptive effect of BV (Sigma, St. Louis, MO, USA; dissolved in 20 \(\mu\)l saline), one of several doses of BV [2.5 mg/kg \((n = 10)\), 0.25 mg/kg \((n = 19)\), 0.25 \(\mu\)g/kg \((n = 14)\), or 0.25 ng/kg \((n = 20)\)] was subcutaneously injected into an acupoint (Zhongwan, CV12) or into a non-acupoint (located in an arbitrary position on the back), 30 min before acetic acid injection. The Zhongwan acupoint was chosen because it is traditionally used for the relief of visceral pain. This acupoint is located in the mid-abdominal area on a line extending between xyphoid process and umbilicus. It should be noted that there is a significant spatial difference between the location of the BV injection site (s.c.) and the acetic acid injection site (i.p).

Two hours after acetic acid injection, 5–8 mice were selected for Fos immunohistochemical analysis. These animals were chosen because their individual behavioral scores approximated the mean of the treatment group. The spinal cord and the brain stem were removed immediately after perfusion, post-fixed in the 4% paraformaldehyde and 0.2% picric acid in 0.1 M phosphate buffer (pH 6.9) for 4–5 h and then placed in 30% sucrose in PBS (pH 7.4) overnight at 4°C. Serial transverse sections (40 \(\mu\)m) of the brainstem and spinal cord were cut using a cryostat (Microm Co.). The tissue sections were processed for Fos immunohistochemistry using the avidin-biotin-peroxidase procedure as previously described [9]. Fos-like immunoreactive (FLI) neurons were visualized using a 3–3 diamino-benzidine (Sigma) reaction. The mean number of FLI neurons per spinal cord region per group was determined by averaging the number of FLI cells in five spinal cord sections (containing the greatest number of FLI neurons from the T6-T13 cord segments) from each animal in the group. All Fos quantitative analysis were performed as previously described [9].

The involvement of opioid and \(\alpha_2\)-adrenergic neurotransmitter systems in BV antinociception were examined by pretreating separate groups of mice with naloxone (Sigma, dissolved in saline) or yohimbine (Sigma, dissolved in distilled water, DW) prior to BV injection. Each antagonist (at a dose of 2 mg/kg) or the appropriate vehicle was injected twice subcutaneously. The first injection was given 5 min before injection of BV (0.25 mg/kg) and the second injection was given 25 min post-injection of BV. Thus, the total dose administered for each antagonist was 4 mg/kg via duplicate injections.

The number of abdominal stretches and the number of FLI neurons in each treatment group were expressed as the mean \(\pm\) SEM. One-way analysis of variance was applied to
analyze the effect of drug treatment on the number of abdominal stretches, as well as the number of FLI neurons in comparison to that of the saline control group. Paired t-tests were used to determine probability values when repeated measures ANOVAs indicated a significant drug effect. A value of $P < 0.05$ was considered to be statistically significant.

BV injection (2.5 mg/kg, 0.25 mg/kg or 0.25 μg/kg) into the Zhongwan acupoint significantly suppressed abdominal stretches in a dose dependent manner (Fig. 1, $P < 0.01$). The antinociceptive effect produced by the three highest doses of BV was not evident when the lowest dose of BV (0.25 ng/kg) was administered. In contrast to acupoint injection, only the highest dose of BV (2.5 mg/kg) produced any detectable antinociception when injected into a non-acupoint (Fig. 1). Based on these results, BV was injected at a dose of 0.25 mg/kg for the pharmacological portion of the present study.

With respect to the Fos expression, the mean number of FLI neurons observed in the spinal cord of naïve animals was $15.5 \pm 3.2$ in the superficial laminae (SDH), $14.2 \pm 2.6$ in the nucleus proprius (NP), $13.2 \pm 2.0$ in the NECK region and $25.4.2 \pm 4.9$ in the ventral horn (VENT). The mean number of FLI neurons was significantly increased in both the spinal cord (Fig. 2) and nucleus tractus solitarii (NTS) in the saline-pretreated + acetic acid injection group (Sal + AA), as compared with the Sal + Sal group (Table 1). Remarkably, BV (0.25 mg/kg) pretreatment (BV + AA) into the Zhongwan acupoint significantly decreased the number of FLI neurons observed in the spinal cord (Fig. 2) and NTS as compared with that of the Sal + AA group (Table 1).

Naloxone pretreatment of acetic acid-injected animals (NX + Sal + AA) had no effect on the number of abdominal stretches when compared to that observed in the saline control group (Sal + Sal + AA), (Table 2). In addition, the analgesic effect of BV pretreatment (Sal + BV + AA) was not antagonized by naloxone pretreatment (NX + BV + AA), (Table 2). Similarly, the α2-adrenergic antagonist yohimbine alone (YOH + Sal + AA) did not change the number of abdominal stretches evoked by acetic acid injection (DW + Sal + AA). However, yohimbine pretreatment (YOH + BV + AA) was found to completely antagonize the reduction in abdominal stretches produced by BV acupoint injection (DW + BV + AA) to the level of the vehicle control (Table 2).

The present study demonstrates that BV injection into the Zhongwan acupoint produces a dose dependent suppressive effect on the abdominal stretch reflex induced by acetic acid injection. Although the highest dose of BV (2.5 mg/kg) tested in the present study produced an antinociceptive effect in the abdominal stretch assay irrespective of site of injection, a lower dose of BV (0.25 mg/kg) only produced antinociception when injected into the acupoint. As a result, the 0.25 mg/kg dose of BV was used for evaluating the mechanism of the BV-induced anti-nociceptive effect throughout the rest of the study.

A large number of studies have now reported that different types of abdominal nociception (including acetic acid-induced abdominal pain and noxious gastric distention) induce Fos expression in both the spinal cord and the NTS [5,15]. It is important to note that the nociceptive behaviors displayed by experimental animals experiencing visceral

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**Fig. 2. Photomicrographs depicting representative examples of Fos expression in the spinal cord of a Sal-Sal animal (A); an acetic acid injected animal (Sal-AA) (B); and an animal injected with bee venom (BV, 0.25 mg/kg) into an acupoint prior to acetic acid injection (C).** An intraperitoneal injection of acetic acid dramatically increased FLI neurons in the spinal cord (B) as compared with that of the control group (A). Pretreatment with BV remarkably reduced the number of FLI neurons in the spinal cord (C) induced by acetic acid injection. The spinal gray matter was divided into the following four regions: (1) SDH, laminae I-II; (2) NP, laminae III-IV; (3) NECK, laminae V-VI; (4) VENT, laminae VII-IX. Scale bars = 250 μm.

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**Table 1**

The effect of BV (0.25 mg/kg) pretreatment on the number of Fos positive neurons in the spinal cord and NTS $^a$

<table>
<thead>
<tr>
<th>Group</th>
<th>Region of spinal cord</th>
<th>NTS</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>SDH</td>
<td>NP</td>
<td>NECK</td>
</tr>
<tr>
<td>Sal + Sal</td>
<td>21.0 ± 2.6</td>
<td>20.0 ± 1.6</td>
<td>17.7 ± 0.5</td>
</tr>
<tr>
<td>Sal + AA</td>
<td>65.0 ± 2.5</td>
<td>65.0 ± 2.5</td>
<td>30.3 ± 6.0</td>
</tr>
<tr>
<td>BV + AA</td>
<td>36.0 ± 3.3**</td>
<td>26.6 ± 3.1*</td>
<td>19.1 ± 2.5</td>
</tr>
</tbody>
</table>

$^a$ The spinal gray matter was divided into the following four regions: superficial laminae (SDH, laminar I-II); nucleus proprius (NP, laminar III-IV); neck region (NECK, laminar V-VI) of the dorsal horn; and the ventral horn (VENT, laminae VII-IX). $^*P < 0.05$ and $^{**}P < 0.01$ as compared with Sal + AA group. $n$ = the number of animals per group. Abbreviations: Sal, saline; AA, acetic acid.
nociception can be correlated with the number of spinal cord neurons that express Fos in these animal models [5,11]. Similarly in the present study, we also observed that the suppression of behavioral responses is closely associated with the reduction of Fos expression in the spinal cord and NTS. Despite the fact that this correlation is similar to previous studies, evaluation of the distribution of spinal cord Fos expression (i.e. SDH, NP, NECK and VENT) in our study shows some differences in comparison to that reported for this type of visceral stimulation in rat [5]. Surprisingly, our results show a much larger increase in Fos staining in the spinal NP region as compared with that reported for visceral or other nociceptive stimuli in rat [5,11]. While this discrepancy could be due to species differences, it is more likely due to other factors including the volume of acetic acid injected in the different studies, the binding affinity and specificity of the Fos antisera or to the exact site of i.p. injection.

The pharmacological portion of our study suggests that BV antinociception may be at least partially mediated by an α2-adrenoceptor associated neural component. The descending noradrenergic system has been both experimentally and clinically shown to modulate the transmission of nociceptive information at the level of the spinal cord [2,7]. Systemic (s.c.) or intrathecal administration of the α2-adrenoceptor agonist, clonidine, produces a dose-dependent elevation of the nociceptive threshold in the abdominal stretch test and this effect is probably mediated by activation of α2-adrenoceptors in the locus ceruleus (LC) and in the dorsal horn of the spinal cord [16]. This hypothesis is further supported by the finding that injection of dexmedetomidine (an α2-adrenoceptor agonist) into the LC produces a dose-dependent increase in the tail-flick latency that is blocked by intrathecal administration of an α2-adrenoceptor antagonist [4]. Based on these findings, adrenergic receptor activation in the LC and spinal dorsal horn ultimately results in the depression of nociceptive transmission from primary afferent fibers to second order nociceptive neurons, thus inhibiting signaling to higher brain regions.

Activation of the descending noradrenergic pain control system has also been observed in previous studies of acupuncture-induced analgesia suggesting that acupuncture stimulation may increase adrenergic activity in the brain stem and spinal cord [6,16]. Based on these findings and the present results, we hypothesize that BV acupoint injection activates the descending noradrenergic system (specifically the α2-adrenoceptor receptor mediated component of this system) and that activation of this system is associated with the antinociceptive effect of BV on acetic acid-induced abdominal stretches.

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Table 2
The effect of the opioid receptor antagonist NX (4 mg/kg) or the α2-adrenoceptor antagonist YOH (4 mg/kg) on BV (0.25 mg/kg) induced antinociception

<table>
<thead>
<tr>
<th>Mechanism</th>
<th>Treatment</th>
<th>No. of abdominal stretch</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>Opioid receptor</td>
<td>Sal + Sal + AA</td>
<td>18.2 ± 1.5</td>
<td>10</td>
</tr>
<tr>
<td></td>
<td>NX + Sal + AA</td>
<td>18.5 ± 1.5</td>
<td>10</td>
</tr>
<tr>
<td></td>
<td>Sal + BV + AA</td>
<td>10.0 ± 1.5**</td>
<td>10</td>
</tr>
<tr>
<td></td>
<td>NX + BV + AA</td>
<td>8.9 ± 0.7**</td>
<td>10</td>
</tr>
<tr>
<td>α2-adrenoceptor</td>
<td>DW + Sal + AA</td>
<td>20.5 ± 4.6</td>
<td>8</td>
</tr>
<tr>
<td></td>
<td>YOH + Sal + AA</td>
<td>17.8 ± 1.6</td>
<td>8</td>
</tr>
<tr>
<td></td>
<td>DW + BV + AA</td>
<td>10.2 ± 1.8**</td>
<td>8</td>
</tr>
<tr>
<td></td>
<td>YOH + BV + AA</td>
<td>17.8 ± 1.6</td>
<td>9</td>
</tr>
</tbody>
</table>

* Each antagonist or its appropriate vehicle (NX: Sal, YOH: DW) was injected at 5 min pre- and at 25 min post-injection of BV. Thirty min after BV or Sal injection acetic acid (AA) was intraperitoneally injected. ** P < 0.01 as compared with vehicle control group (bold font). Abbreviations: Sal, saline; DW, distilled water.


